Studies on Molecular and Traditional Diagnostic Assays for *Listeria monocytogenes* Infection in Sheep

Soumaya S. A. El – Shafii and Omnia F. H. Shalby
Bacteriology Department – Animal Health Research Institute

Abstract

L. monocytogenes **has been to be of world-wide prevalence and associated with serious disease in a wide variety of animals and man. It is an exquisitely adaptable environmental bacterium capable of existing both as animal pathogen and plant saprophytic. *Listeria monocytogenes* is associated with meningoencephalitis, septicaemia and abortion in sheep, other ruminants and humans. The study population consisted of 3 sheep farms (No. of herd ranged from 150-250 animals) located in different area in Egypt including 2 cases and one farm with no symptoms. The most clinical symptoms were neurological signs, including circling, excessive salivation and unilateral paralysis, septiceamia and diarrhea. Approximately 50 samples were collected from each farm including 40 fecal and 10 from internal organs of five dead lambs including brain, spleen, liver and intestine. Farm (2) showed that highest rate of infection (92.5% in feces and 90% from internal organs) followed by farm (1) in which the rate was 55% and 70% respectively. On the other hand the 3rd farm showed the isolation of *L.monocytogenes* from fecal samples only in a rate of 35%. PCR results of the PCR assays with the primer combination LL5 and LL4 on DNA extracted from 9 *L.monocytogenes* isolates showed a band at 520bp. These were of the size predicted based on the published nucleotide sequence of the listeriolysin O gene for the respective primer combination. *L.monocytogenes* was detected in representative samples from the 3 farms, and it was found that 1 sample of farm (3) was negative on conventional method but it was positive in PCR. Pathogenicity test in mice revealed the pathogenic and high virulence nature of the isolates. It was concluded that monitoring of *L.monocytogenes* infection in sheep and other farm animals is important as carrier play a role in spread of infection to animals and human. PCR assays on DNA extracted from isolated or from clinical specimens provide a rapid, sensitive and specific method for detection of *L.monocytogenes*.The procedure takes not more than 72h to complete and detect the infection and this consume time of diagnosis.**

Introduction

Listeria is a genus of bacteria named after Lord Joseph Lister, the English surgeon and medical researcher. These bacteria appear as regula: short rods of about 0.4 – 0.5 um in diameter and 0.5 – 2.0um in length with rounded ends. They are gram positive, but not acid fast. They do not form capsule or spores (**23**).

The facultative intracellular gram positive bacterium *L. monocytogenes* is a food–born pathogen of frequently underestimated importance (**2**).

Following the initial isolation and description in 1926, *L. monocytogenes* has been to be of world-wide prevalence and associated with serious diseas in a wide variety of animals and man. It is an exquisitely adaptable environmental bacterium capable of existing both as animal pathogen and plant saprophytic. *Listeria monocytogenes* is associated with meningo-encephalitis, septicaemia and abortion in sheep, other ruminants and humans (**10**).

It was observed that a single *L. monocytogenes* strain can lead to infection of multiple animals with rapid progression of the disease (**24**).

Recent attention has focused on rapid detection systems for *L. monocytogenes*. While cultural methods provide the "gold standard", these are labor intensive and time consuming. A more rapid method which specifically identifies *L. monocytogenes* is required. DNA probes complementary to the listeriolysin O gene [hly A] (**18**), also hybridization studies on lap locus, dth gene have been successful (Lampel et al., 1990). Recently polymerase reaction (PCR) assays based on the amplification of target DNA sequences in the listeriolysin O (**4; 5 and 6**) and dth genes have been reported.

The aim of the present study is isolation and identification of *L. monocytogenes* from sheep suffering from nervous manifestation and applied a trial for rapid diagnosis of *L. monocytogenes* in clinical specimens using PCR.

Material and Methods

Sheep listeriosis case farms enrolled in this study was identified. Clinical listeriosis cases were defined as cases meeting one or more of the following criteria: (1) isolation of *L. monocytogenes* from specific organs obtained from dead lambs by necropsy; (2) veterinary diagnosis of clinical listeriosis symptoms.

The study population consisted of 3 sheep farms (No. of herd ranged from 150-250 animals) located in different area in Egypt including 2 cases and one farm with no symp oms. Sample collection took place from 2005-2006. The most clinical symptoms were neurological signs, including circling, excessive salivation and unilateral paralysis, septiceamia and diarrhea.

Approximately 50 samples were collected from each farm including 40 fecal and 10 from internal organs of five dead lambs including brain, spleen, liver and intestine. Each sample was collected into a sterile bag by the use of clean gloves. All samples were stored in clean coolers with ice bag for transit to the laboratory. Samples were processed within 24h of collection.

Fecal samples and organs (10g and 25g respectively) were aseptically transferred into sterile bags and listeria enrichment broth was added to achieve a 1:10 dilution. They were homogenized manually for 1 min until solid matter was completely suspended in the enrichment solution. Enrichment broth was incubated at 30°C for 24-48h.

50ul aliquots of the enrichment broth was plated onto Oxford medium and incubated for 48 at 30°C and observing the black halos **(17)**. Isolated colonies were identified morphologically and biochemically according to **(10)** depending on beta-hemolysis on sheep blood agar, motility, nitrate reduction,catalase test, sugar fermentation and CAMP tests.

Isolation of genmomic *L. monocytogenes* DNA:

Nine *L. monocytogenes* isolates were chosen randomly to represent the three farms. DNA was extracted from bacteria grown in brain heart infusion broth (100ml) and incubated overnight at 37°C. The cells were harvested by centrifugation and suspended in 7ml of 25% sucrose-50mM EDTA- 50mM Tris HCL (pH 8). Lysozyme was added to a final concentration of 0.5mg/ml and incubated for 45min at 37°C, 35 ml of lysis buffer (10mMTris HCL

[pH8], 1mM EDTA, 1% sodium dodecyl sulfate, 5µg of freshly prepared pronase per ml) was added and incubation was continued at 37°C for 30 min. two phenol-chloroform-isoamyl alcohol (50:90:1) extraction and one chloroform-isoamyl (25:1) extraction was followed. DNA was precipitated by addition of 2.5 volumes of ethanol, washed, and redissolved in 400ul of 10mM Tris HCL-1mM EDTA (pH 7.5) to a final concentration of approximately 1ug/ul **(19).**

Extraction of DNA from clinical specimens:

Four clinical specimens (brains) positively *L. monocytogenes* on convential methods and two clinical specimens (brains) negative to *L. monocytogenes* on convential methods were used to be submitted for PCR as a molecular diagnosis. A 25g was blended in a stomacher in 225ml of Palcm-listeria-enrichment broth for 2min and incubated at 37°C for 48h.one ml of enriched samples were centrifuged at 13,000g for 10min. The pellets were washed twice in sterile water, then 5µl of the suspension was recovered in 200µl lysis reagent (12.5µl protease to 1ml lysis buffer) and heated at 55°C for 1h, then 95°C for 10min., after cooling, 50µl of lysed samples from lysis tube were transferred into the PCR tubes **(3).**

Table (1): Nucleotide sequence of oligonucleotide primers

Primer	Orientation	Sequence (5`-3`)	G+C Conc.	Location within gene (bp)*
LL_5	Forward	AAC CTA TCC AGG TCC TC	53	372-389
LL_4	Reverse	CCC CAC ACT TGA GAT AT	47	874-891

- From published hlyA gene for listeriolysin O **(15).**

PCR assays:

PCR assays were performed in 100µl volumes containing 2-3mM $Mgcl_2$;10mM Tris HCl (pH 8.3); 50mM KCl; 1ng of template DNA, 0.2mM each dATP, dGTP, dCTP and dTTP, 1mM of each primer and 2.5 U of Taq DNA polymerase.

PCR assays were done on bacterial DNA with forward and reverse primer combinations in DNA thermal cycler by using 30 cycles of 1min at 94°C, 1 min at 55°C and 2 min at 72°C and a final incubation at 72°C for 5 min.

PCR products were analyzed by agarose gel electrophoresis by using 1.2% gels containing ethedium bromide (7).

Pathogenicity test:

Seven group of white mice (10 mice in each group). Six groups were inoculated intraperitoneal with 0.25µl of isolated *L.monocytogenes* broth culture and the seventh group was remained as control group (8 and 22).

Two strains of *L.monocytogenes* were chosen randomly from each infected farm and used for the pathogenicity tests. Group 1 and 2 represent farm 1, groups 3 and 4 represent farm 2, groups 5 and 6 represent farm 3and group 7 was considered as control group. The 6 strains were confirmed identified by PCR assays.

Results

Isolation and identification of *L.monocytogenes*:

Suspected colonies showed narrow zone of B-haemolysis on sheep blood agar and black colonies on Oxford media, with hallo zone. These isolates were Gram positive coccobacilli and catalase positive, showed synergestic haemolysis with *S. aureus* (B-toxin producing strain) on CAMP test. The isolated strains were motile, using hanging drop preparation showing the tumbling movement type. The suspected colonies ferment dextrose, L-rhaminose and variable with lactose and not ferment manitol and D-xylose.

Table (2) revealed the occurrence of *L.monocytogenes* in three farms, where farm 2 showed that highest rate of infection (92.5% in feces and 90% from internal organs) followed by farm (1) in which the rate was 55% and 70% respectively. On the other hand the 3rd farm showed the isolation of *L.monocytogenes* from fecal samples only in a rate of 35%.

Table (2): Occurrence of *L.monocytogenes* in different samples collected from 3 farms

Farm	Type of sample	No. of sample	Occurrence of *L.monocytogenes*		Negative samples	
			No.	%	No.	%
Farm 1	feces	40	22	55	18	45
	Internal organs	10	7	70	3	30
Farm 2	feces	40	37	92.5	13	32.5
	Internal organs	10	9	90	1	10
Farm 3	feces	40	14	35	26	65
	Internal organs	10	0	0	0	0

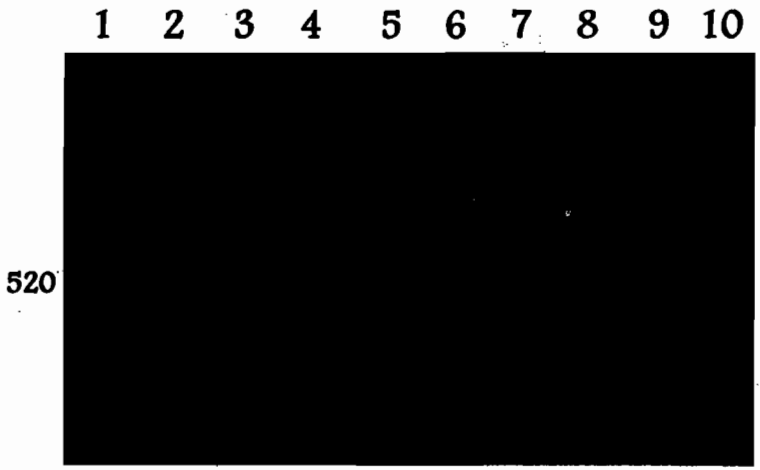

Fig. (1): PCR assay of *L.monocytogenes* isolates. Lane 1 is a marker, lane 2 is negative control lanes 3-10 are the isolated *L.monocytogenes*.

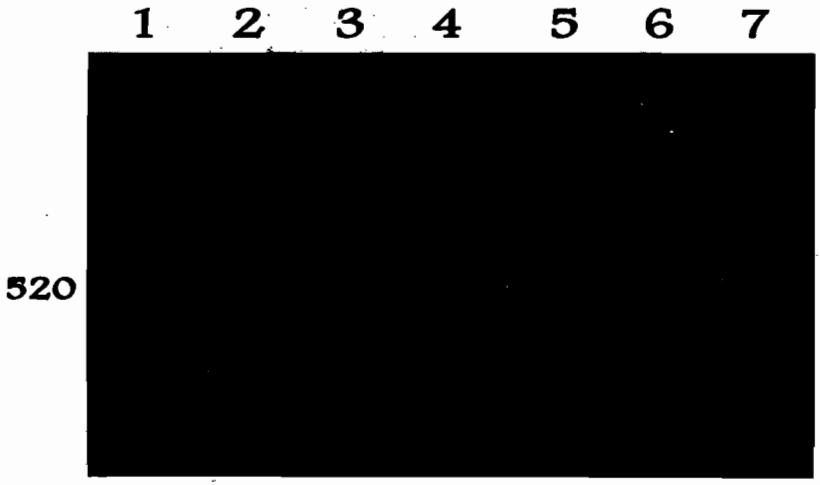

Fig. (2): PCR assay of *L. monocytogenes* in clinical specimens. Lane is a marker, lane 2 is negative control, lanes 3,4,6,7 are positive clinical specimens and lane 5 negative clinical specimen

PCR assays:

PCR results of the PCR assays with the primer combination LL5 and LLA on DNA extracted from 9 *L.monocytogenes* isolates are shown in Fig. (1), where all template samples showed a band at 520bp. These were of the size predicted based on the published nucleotide sequence of the listeriolysin O gene for the respective primer combination.

Fig. (2) represents the PCR assays with the primer combination LL5 and LLA on DNA extracted from clinical specimens, 5 out of 6 templete DNA samples showed a band at 520 bp., while the sixth sample was negative.

Pathogenicity test in mice:

All inoculated mice were died within 24-72h except in groups (1-4), while groups (5-6), 7 mice from each group was died and group 7 showed no death.

L.monocytogenes was reisolated from liver, spleen and brain of dead mice, macroscopically, liver showed congestion and focal areas of necrosis through the hepatic parenchyma.

Discussion

Interestingly, sheep are seen to be more susceptible to *L.monocytogenes* than cattle (24).

Results of this study indicate the oocurrence of *L.monocytogenes* in different farms. Farm (2) was at a risk of developing listeriosis where the rate of isolation was reported in this farm (92.5% from feces and 90% from internal organs of dead ones). In farm (3), although there was no evidence of clinical nervous symptoms, *L.monocytogenes* was isolated from 14 fecal samples (35%). These results revealed the presence of carriers and under risk factors or environmental factors spread of infectrion will release through fecal shedding. These results agree with (16) and (12).

The culture characters of isolated strains, morphological characters and their biochemical activities obtained in this study were similar to that reported by (21) and (23).

In this study, PCR assays were used for two purposes firstly confirmed the convential identification of *L.monocytogenes* isolates, second diagnosis of *L.monocytogenes* in clinical specimens of sheep as a ranid technique.

PCR assays showed that all isolated strains were *L.monocytogenes* as it showed a band at 520 bp., these results agree with **(19)** and **(7)**. On the other hand, Fig. (2) revealed the presence of *L.monocytogenes* in representative samples from the 3 farms, where 1 sample of farm (3) was negative on conventional method but it was positive in PCR. This result revealed that conventional methods is not quit enough to diagnose *L.monocytogenes* and in need to apply more advanced technique as PCR for diagnosis specially when monitoring for carrier. These results were agreed with **(9)**.

Presence of *L.monocytogenes* in fecal samples in farm (3) pointed to apply pathogenicity test to measure the pathogenicity and virulence of isolated strains in comparison to that isolated from the other 2 farms. The death of all inoculated mice in groups (1-4) and in 7 mice of groups (5-6) with septiceamic lesions and reisolation of *L.monocytogenes* proved the pathogenic and high virulence nature of the isolates. These results were in agreement with **(1)**; **(11)** and **(22)**.

It was concluded that monitoring of *L.monocytogenes* infection in sheep and other farm animals is important as carrier play a role in spread of infection to animals and human.

PCR assays on DNA extracted from isolated or from clinical specimens provide a rapid, sensitive and specific method for detection of *L.monocytogenes*. the procedure takes not more than 72h to complete and detect the infection and this consume time of diagnosis.

References

1. **Abd El-Wanees, Soad Abdel Al-Aziz (1985):** Studies on listeriosis in rabbit M.V.Sc. Thesis, Fac. Vet. Med. Cairo. Univ.

2. **Abram, M. and Dorie, M. (1997):** Primary *Listeria monocytogenes* infection ingestating mice. Folia-Microbiology (Praha), 42; (1): 65-70.

3. **Beker, B.; Jordan, S. and Holzapfel, W.H. (2005):** rapid and specific detection of *Listeria monocytogenes* in smoked salmon with BAX-PCR. J. Food Control, 16: 717-721.

4. **Bessesen, M. T. Q. Luo; Rotbart, H. A.; Blaser, M. J. and Ellison, R. T. (1990):** Detection of Listeria monocytogenes by using the polymerase chain reaction Appl. Environ. Microbiol., 56: 2930-2932.

5. **Border, P. M.; Howard, J. J.; Plastow, G. S. and Siggens, K. W. (1990):** Detection of listeria species and Listeria monocytogenes by using the polymerase chain reaction. Lett. Appl. Microbiol., 11: 158-160.

6. **Denser, H. G. and Boychuk, I. (1991):** Species-specific detection of listeria monocytogenes by DNA amplification. Appl. Environ. Microbiol., 57: 606-609.

7. **Elizabeth, J.; Thomas, G.; King, R.K.; Burchark, J. and Gannon, V.P.J. (1991):** Sensitive and specific detection of Listeria monocytogenes in milk and ground beef with the polymerase chain reaction. Appl. Environ. Microbiol., 57; (9): 2571- 2580.

8. **Emmerling, P.; Finger, H. and Bochemuhl, J. (1975):** *Listeria monocytogenes* infection in Nude mice. Infec. Immun., 12; (20): 437-439.

9. **Franciosa, G.; Tartaro, S.; Wedell-Neergaard, C. and Aureli, P. (2001):** characterization of *Listeria mopnocytogenes* strains involved in invasive and non invasive listeriosis outbreaks by PCR-based Fingerprinting techniques. Appl. Environ. Microbiol., 67; (4): 1793-1799.

10. **Gray, M. L. and Killinger, A. H. (1966):** Listeria monocytogenes and listeric infections. Bacteriol. Rev., 30: 309-382.

11. **Hassanein A.R. (1994):** Epidemiological studies on the occurrence of listeria infection in animal and man M. V. Sc. Thesis Fac. Vet. Med. Assuit Univ.

12. **Ho AJ.; Jvanek R.; Gröhn YT.; Nightingale K.K. and Wiedmann M. (2007):** *Listeria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific *Listeria monocytogenes* subtypes. Prev. Vet. Med., 80; (4): 287-305.

13. **Lampel, K. A.; Jogow, J.A.; Tuckess, M. andHill, W. E.(1990):** polymerase chain reaction for detection of invasive Shigella flexneri in food. Appl. Eniron. Microbiol., 56:1536-1540

14. **Low, JC and Donachie, W. (1997):** A review of *Listeria monocytogenes* and listeriosis Vet.J. 153; (1): 9-29.

15. **Mangaud, B.; Vincente, M.F.; Chenevert, J.; Pereira, C.; Geaffroy, C.; Giequel-Sanzey, B.; Baquero, F.; Perez, J.C. and Cossart, P. (1983):** Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes.* Infect. Immun., 56:766-772.

16. Nightingale K.K.; Fortes E. D.; Ho AJ.; Schukken Y. H.; Gröhn YT. and Wiedmann M. (2005): Evaluation of farm management practices as risk factors for clinical listeriosis and fecal shedding of *Listeria monocytogenes* in ruminants. J. Am. Vet. Med. Assoc., 227; (110: 1808-1814.

17. Nightingale, K. K.; Schukken, Y. H.; Nightingale, C. R.; Fortes, D.; Ho, A. J.; Her, Z.; Grohn, Y. T.; Mc Donough, P. L. and Wiedmann, M. (2004): Etiology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Applied and Environmental Microbiology, 70; (8): 4458-4467.

18. Peterkin, P. I.; Idziak, E. S. and Sharpe, A. N. (1991): Detection of Listeria monocytogenes by direct colony hybridization on hydrophobic grid-membrane filters by using chromagen-labeled DNA probe. Appl. Environ. Microbiuol., 57: 586-591.

19. Rasmussen, O. F.; Beck, T.; Olsen, J. E.; Dons, L. and Rossen, L. (1991): *Listeria monocytogenes* isolates can be classified into teo major types according to the sequence of listeriolysin gene. Infect. Immun., 59; (11): 3945-3951.

20. Seeliger, H. P. R. and Jones, D. (1986): Bergey's Manual of Systemic Bacteriology William and Wilkins, Baltimore. MD.

21. Sneath, P. H. A.; Mair, N. S.; Sharpe, M. E. and Holt, J. G. (1986): Bergey's Manual of SystemicBacteriology. Vol. 2, pp. 1235-1244. Wiliams and Wilkins, Baltimore-Hongkong. London.

22. Soumaya S. A. S. and Amer H.A. (2002): An attempt to control the major manifestations (abortion and nervous symptoms) attributed to *Listeria monocytogenes* infection in sheep. Vet. Med. J., 50; (4):639-656.

23. Topcly and Wilson (1998): Microbiological and microbial infection. 9th Ed. Vol. 1 (Systemic Bacteriology) Oxford Univ. Press USA.

24. Wiedmann, M.; Arivik, T.; Bruce, Jl.; Neubaner, J.; delPiero, F.; Smith, Mc. Hurley, J.; Mohammed HI and Batt CA (1997): Investigation of a listeria epizootic in sheep in NewYork State. Am. J. Vet. Res., 58; (7): 733-737.

دراسات عن التقنيات الحديثة والطرق التقليدية لتشخيص الاصابة بميكروب الليستريا مونوسيتوجين فى الخراف

سمية السيد أحمد الشافعى ـ أمنية فكرى حسن شلبى
قسم البكتريولوجى- معهد بحوث صحة الحيوان- الدقى

الملخص العربى

تم دراسة ميكروب الليستيريا مونوسيتوجين فى ثلاث مزارع للاغنام يتراوح العدد الكلى بين ١٥٠-٢٥٠ غنم بالمزرعة بمناطق مختلفة بمصر. وكانت ،زرعتان بهما الاعراض مرضية ومزرعة ليس بها أى أعراض، و كانت هذه الاعراض المرضية عبارة عن أعراض عصبية والدوران وكذلك زيادة فى افراز اللعاب وشلل نصفى واسهال. تم جمع ٥٠ عينة تقريبا (٤٠ عينة براز ،١٠ عينات من الأحشاء الداخلية) لخمس حملان نافقة وتشمل هذه العينات الخ والطحال والكبد والامعاء. وقد تم عزل ميكروب الليستريا مونوسيتوجين بأعلى نسبة من المزرعة (٢)، حيث كانت النسبة ٩٢,٥% من عينات البراز ، ٩٠% من عينات الأحشاء. بينما كان معدل العزل فى المزرعة (١) أقل (٥٥% ،٧٠ على التوالى). ومن ناحية أخرى تم عزل الميكروب من عينات البراز فى المزرعة (٣) بنسبة ٣٥%.

وكانت نتائج اختبار البلمرة (بى سى أر) للعترات المعزولة (٩ عترات)أظهرت الشريط عند الوزن ٥٢٠ بيز بير وكذلك تم التعرف عن ميكروب الليستيريا مونوسيتوجين فى العينات الممثلة للاحشاء الداخلية، وقد وجد ان هناك عينة ايجابية للميكروب وكانت سلبية بطريقة الزرع. وباجراء اختبار احداثية الاصابة فى الجرذان، وجد ان معظم العترات المعزولة التى تم اختبارها ذات ضراوة عالية ولها القدرة على احداث النفوق فى الجرذان.

وقد أوضحت هذه الدراسة الى ايجابية الفحص الدورى للمزارع للكشف عن الغنام الحاملة لهذا الميكروب دون أعراض للحد من انتشار المرضوتحديد كيفية التحكم فى مقاومته. وكذلك وجد ان اختبار البلمرة (بى سى أر) ذات حساسية وكفاءة عالية وكذلك اختبار سريع لتشخيص ميروب الليستيريا مونوسيتوجين فى العينات المختلفة حيث أخذ الاختبار مدة لا تزيد عن ٧٢ ساعةلتشخيص هذا الميكروب.